Effects of pH and Temperature on the Enzymatic Hydrolysis of Crop Residues by Fungal Cellulases

Caroline Mariana de Aguiar, Alessandra Rodrigues Rufino, Salah Din Mahmud Hasan, Sérgio Luiz de Lucena

Abstract— Lignocellulosic materials such as the crop residues are abundant in the world and they can be an important source of cellulose for bioprocesses such as the production of cellulosic ethanol. Cellulose is a biopolymer formed by glucose units that can be hydrolysed by cellulase enzymes under specific physical and chemical conditions. The pH and temperature play a very important role on the enzymes activity. Cellulases were used for hydrolysing the sugarcane bagasse, corn straw and wheat straw by applying different pH and temperature values during the enzymatic hydrolysis. The aim of this work was to evaluate the effects of pH and temperature on the enzymatic hydrolysis of the crop residues sugarcane bagasse, corn straw and wheat straw by using cellulases produced by *Aspergillus niger*. It was observed that both the pH and the temperature T affect the cellulases activity. It was concluded that by applying the pH=4.8 and T=50ºC yielded the maximum cellulase activity.

Index Terms— biomass, cellulase, cellulose, enzyme, corn straw, lignocellulosic biomass, sugarcane bagasse, wheat straw

1 Introduction

Lignocellulosic materials are produced by plants as part of their constitutive biomass being the most abundant agro-industrial residues in the world. Due to its cellulose content, they can be an important source of carbohydrates for many bioprocesses. Recently, the lignocellulosic biomasses have gained increasing research interests and special importance because of their renewable nature as a raw material for the production of bio-ethanol. The development of new fermentation processes utilizing lignocellulosic biomass as a raw material for biofuel production can minimize the world´s dependence on fossil fuels by providing a convenient and renewable source of glucose (Ojumu, 2003). Brazil has a wide variety of agricultural and agro-industrial residues and the processing of those wastes may be of great economic, social and environmental interest (Lima et al., 2007). There are plenty of waste materials derived from activities such as pulp and paper industries, wood processing, production of ethanol and sugar from sugarcane, agricultural crop production of cereals, fruits, amongst others (Ramos, 2000).

Lignocellulosic materials are composed of cellulose, hemicellulose, lignin and minor amounts of extractives (Tamanini, 2004). The cellulose is a biopolymer formed by glucose units linked by β-(1-4) glycosidic bonds. The cellulase enzymes can release those glicose molecules from the cellulose chain. The cellulose structure has some crystalline regions that present more resistence to enzymatic hydrolysis. The less ordered, amorphous areas where the chains have random orientation are more susceptible to enzyme activity (Gupta, 2008). The adjacent cellulose chains form a set of aggregates, or elementary fibrils, that are associated with each other to form the cellulose crystallite. Subsequently, four of those aggregates are joined by a monolayer of hemicellulose and lignin. The natural compound that results from this association is called cellulose microfibril (Pitarelo, 2007). The association of different types of polymers that comprise the vegetable matter (cellulose, hemicellulose and lignin), the degree of crystallinity and the packaging caused by the complex structure of lignin form a rigid structural material that is naturally very resistant to the enzymatic activity. This characteristic makes being necessary some pretreatments, already in the early stages of bioconversion of the lignocellulosic biomass to ethanol (Gupta, 2008). The pretreatments aim to remove most of the lignin and hemicellulose barriers. Also, the pretreatments reduce the cellulose crystallinity and increase the porosity of the lignocellulosic biomass and thereby exposing the cellulose to the enzymatic action. There are several types of physical and chemical pretreatments that can be applied in the lignocellulosic biomass wich increase the susceptibility of cellulose to enzymatic hydrolysis (Kumar et al., 2009). An alkaline chemical pretreatment is performed using solutions of NaOH, $Ca(OH)_2$ or NH_3. It removes part of the lignin and part of the hemicellulose wich improves the accessibility of the cellulase enzymes. The alkaline treatment is considered being very effective in breaking the bonds between cellulose, hemicellulose and lignin, and promote the fragmentation of hemicellulose (Taherzadeh and Karimi, 2008). De Aguiar et al. (2017) compared the effects of alkaline and alkaline-oxidative pretreatments of the sugarcane bagasse, corn straw and wheat straw on their cellulose content and on the enzymatic hydrolysis using cellulases produced by the fungus *Aspergillus niger*.

Several microorganisms can produce cellulase enzymes. Cellulases are able to hydrolyze the cellulose chain producing low molecular weight sugars like glucose and cellobiose (Martins, 2005). Muthuvelayudham and Viruthagiri (2006) cultivated the fungus *Trichoderma reesei* to produce cellulases using

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sugarcane bagasse and rice straw as fermentation substrates. Ojumu et al. [1] (2003) used the fungus *Aspergillus flavus* to produce cellulases using powder-saw, sugarcane bagasse and corn cobs as the fermentation substrates.

The set of enzymes that is involved in the hydrolysis of cellulose is called cellulasic enzyme complex. According to Lynd and Zhang (2002) such complex is divided into three groups, according to their place of actuation in the cellulosic substrate:

1) Endoglucanases: the enzymes that ramdomly hydrolyze the internal regions of the amorphous structure of the cellulose chain and produce oligosaccharides and, consequently, new reducing and non-reducing terminals;
2) Exoglucanases: the enzymes that are divided into cellobiohydrolases (CBHs) and glucanohidro-lases (GHs). The CBHs are responsible for releasing cellobiose from the cellulose ends. The GHs are able to release glucose directly from the cellulose chain.
3) β-glycosidases: convert cellobiose and the soluble oligosaccharides (with less than seven monomeric units) into glucose.

Aspergillus niger is a fungus commonly found in nature. It is grown in many fermentation processes for the production of some enzymes and organic acids under specific physical and chemical conditions. *Aspergillus niger* produces cellulases and It may be considered superior to other fungi that are known being good producers of the cellulasic complexes, such as *Trichoderma reesei* (Aguiar and Menezes, 2000).

Enzymes are biomolecules (typically proteins) that present a very specific catalytic activity transforming substrates in products. Proteins are formed by peptide bonds between amino acids resulting in a long polypeptide chain that presents a primary, a secondary and, a tertiary structure. Some proteins exhibit a quaternary structure depending on the solution´s pH. The structures are originated from chemical interactions between the amino acid residues along the polypeptide chain. The enzyme´s structures play a very important role on the enzyme activity, more specifically the structure of the active site. It is in the active site where the enzyme-substrate interaction effectively ocurrs to form the products. The three-dimensional 3D structure of the enzymes produces a suitable and specifically shaped catalytic active site for interacting with a substrate and forming products under appropriate physicochemical conditions. The temperature and the pH are important factors that can affect the enzyme structures and, consequently, the enzyme-substrate interaction. The rate of enzyme catalyzed reaction increases with temperature up to a certain limit. Above a certain temperature, enzyme activity decreases with temperature because of enzyme denaturation. Then we can observe an optimal temperature where the enzyme activity is maximum (Shuler and Kargi, 2001). The pH affects the net electrical charge of the enzyme molecule and, consequently, the 3D structure of the active site when ionic groups are present. The changes in the active site affect its kinetics properties and enzyme activity. Most of the enzymes have a pH condition that maximize the enzyme activity (optimum pH). The knowledge of the enzyme´s optimum pH is paramount for any enzymatic process.

This study aimed to evaluate the effects of pH and temperature on the enzymatic cellulase activity in the hydrolysis of the pretreated crop residues sugarcane bagasse, corn straw and wheat straw. The cellulases were produced when growing *Aspergillus niger* using sugarcane bagasse as the fermentation substrate.

2 Material and Methods

2.1 Lignocellulosic substrates

It was used sugarcane bagasse, corn straw and wheat straw as lignocellulosic materials. Sugarcane bagasse was kindly supplied by COOPCANA (Cooperativa Agrícola Regional de Produtores de Cana) located in Paraíso do Norte, PR, Brazil. Corn straw and wheat straw were collected in the plantation fields soon after harvesting.

2.2 Physical pretreatments

The wheat straw and corn straws were sun dried and milled in a Trapp hammer mill, model TRF-400, and then sieved in No.4 mesh. The sugarcane bagasse was sun dried only. All dried and powdered lignocellulosic substrates were stored in sealed plastic bags and kept in the fridge for later use.

2.3 Chemical pretreatments

The alkaline treatment was carried out according to the procedure described by Aguiar and Menezes [13]: after the physical pretreatment, the lignocellulosic substrates were immersed in a 4% (wt/wt) NaOH solution and autoclaved at 121°C during 30 min. and then they were extensively washed with tap running water. Phosphoric acid was added until neutral pH was reached. The washed, now alkaline treated substrates were oven dried at 65 °C.

2.4 Fungal cellulases production by fermentation

The fermentation was carried out in 2000 mL Erlenmeyer flasks that were autoclaved at 120 °C during 20min. The fermentation solid substrate was the alkaline treated sugarcane bagasse (100 g/L) as the main carbon source and then using 1000mL of culture medium described by Mandels and Weber [15]. Tween 80 (1mL/L) was added to the culture medium and the flasks were inoculated with 10mL of *Aspergillus niger* spores suspension (about $1x10^6$ spores/mL) and incubated at 30°C during 7 days as described by de Aguiar and de Lucena [16]. The fermentation broth was recovered by filtration and the liquid fraction containing the cellulases (enzyme extract) was used for the enzymatic hydrolysis of the cellulosic substrates.

2.5 Enzymatic hydrolysis of the cellulosic substrates

The enzymatic hydrolysis of the sugarcane bagasse, wheat straw and corn straw (cellulosic substrates) were based on adapted methodology described by Ghose (1987). In triplicated assay tubes were added 300mg of each the chemically pretreated cellulosic substrate, 4mL of the buffer solution at selected pH, and 2mL of the enzyme extract. The tubes were incubated at the selected temperature for 50 min. The effects of temperature were carried out at pH=4.8 and the effects of pH were carried out at T=50°C. The total reducing sugars (TRS) released during hydrolysis were measured according the methodology described by Miller (1959). It was defined that one unit

of cellulase activity (1U) releases 1µmol of TRS by 1 mL of the enzyme extract by 1 minute (1U = 1 µmol mL^{-1} min^{-1}) using the cellulosic substrates.

2.6 Buffer solutions

It was prepared 50mM acetate buffer pH=2.9, pH=3.8, pH=4.8 and, pH=5.9, and 50mM phosphate buffer pH=6.7, pH=7.8 and, pH=8.8. A pHmeter was used for pH measurements.

3 Results and Discussions

After the physical pretreatment the sugarcane bagasse, wheat straw and corn straw were submitted to the alkaline chemical pretreatment prior their hydrolysis by the fungal cellulases.

Effects of the temperature on the enzymatic hydrolysis. The figure 1 shows the results when different temperatures (ranging from 20°C to 80°C) were applied during 50min for the enzymatic hydrolysis of the cellulosic substrates by using 50mM acetate buffer at pH=4.8.

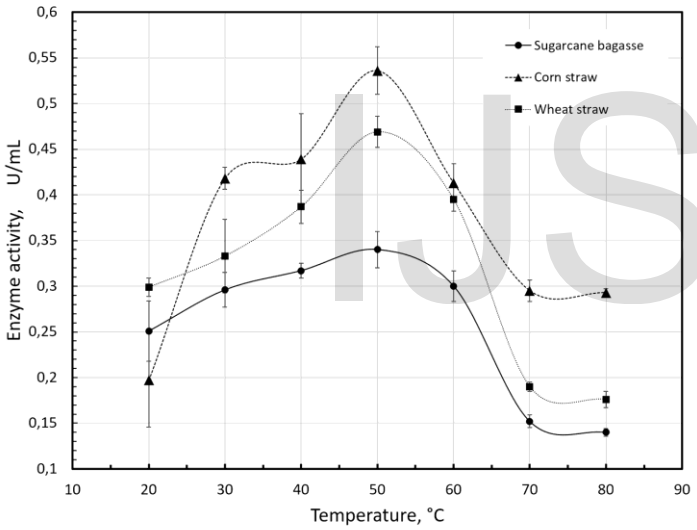

Fig.1. Effect of temperature on the cellulase activity for the enzymatic hydrolysis of sugarcane bagasse, corn straw and wheat straw using 50mM acetate buffer at pH=4.8.

We can observe that as the temperature increases there is a favourable effect by gradually increasing the enzyme activity for all the lignocellulosic substrates. The enzyme activity then reaches its peak when temperature is 50°C being 0.340U/mL for sugarcane bagasse, 0.536U/mL for corn straw and 0.469U/mL for wheat straw. Hence, there is a thermal activation of the enzymes up to 50°C. As the temperature increase from beyond 50°C we observe a negative effect on the enzyme activity and the enzymes molecules are being deactivated due to the thermal energy applied. The energy reaches values higher enough that affect negatively the interactions that maintain the effective structure of the active sites. The active sites rapidly loose their ideal 3D conformation to interact with the cellulosic substracts and carry out the hydrolysis causing a decrease in the enzyme activity.

Effects of the pH on the enzymatic hydrolysis. The figure 2 shows the results when different pH values (ranging from 2.9 to 8.8) were applied during 50min for the enzymatic hydrolysis of the cellulosic substrates at 50°C.

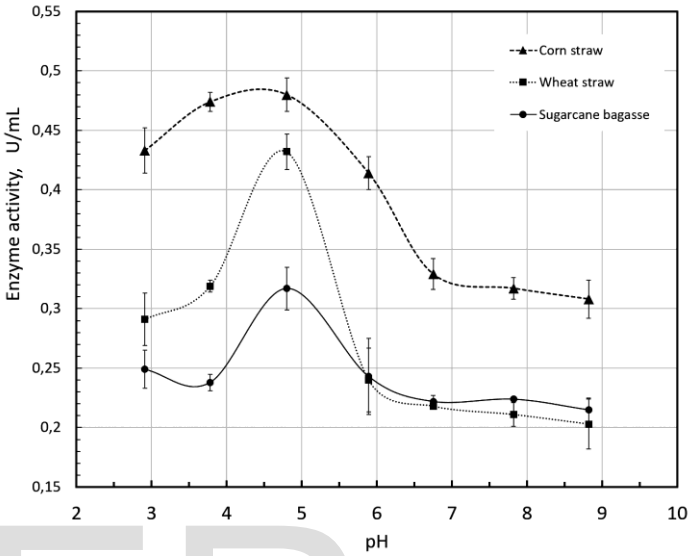

Fig.2. Effect of the pH on the cellulase activity for the enzymatic hydrolysis of sugarcane bagasse, corn straw and wheat straw at 50°C.

The results show that the pH is an important factor that affects the cellulase enzyme activity. We can observe in the figure 2 that there is a narrow range of pH where the enzyme activity reaches higher values. It is around pH=4.8 where the cellulase activity peaks for all the lignocellulosic substrates being 0.317U/mL for sugarcane bagasse, 0.480U/mL for corn straw and 0.432U/mL for wheat straw. Enzymes are amphoteric molecules containing a large number of acid and basic groups. The charges on these groups will vary, according to their acid dissociation constants, with the pH of their environment. This will affect the total net charge of the enzymes and the distribution of charge in the molecule, in addition to the reactivity of the catalytically active groups. These effects are especially important in the neighbourhood of the active sites. Then, the changes in charges with pH affect the activity and structural stability of the enzyme. Hence, the pH can affect the ionic interactions that exist in the active site and keep a favourable configuration to bind to the cellulosic substract and then carrying out its hydrolysis.

4 Conclusion

We concluded that both the temperature and the pH are important factors that affect the hydrolysis of the cellulosic substrates sugarcane bagasse, corn straw and wheat straw by using cellulases produced by *Aspergillus niger*. It was observed

that the temperature 50°C and the pH=4.8 yielded the maximum cellulase activity for the enzymatic hydrolysis of sugarcane bagasse, corn straw and wheat straw.

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